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Intranasal Vaccination of Humans with Recombinant Cholera Toxin B Subunit Induces Systemic and Local Antibody Responses in the Upper Respiratory Tract and the Vagina

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Forty-five volunteers were vaccinated twice intranasally with 10, 100, or 1,000 μg of cholera toxin B subunit (CTB). Blood and nasal and vaginal secretions were collected before and 1 week after the second vaccination from all volunteers, and the specific and total immunoglobulin A (IgA) and IgG titers were determined by enzyme-linked immunosorbent assay. Samples were also taken 6 months ($n = 16$) and 1 year ($n = 14$) after the vaccination. The 10- and 100-μg doses were well tolerated by the volunteers, but the 1,000-μg dose induced increased secretions from the nose and repetitive sneezings for several hours. The CTB-specific serum IgA and IgG increased 21- and 7-fold, respectively, 1 week after vaccination with the medium dose and increased 61- and 37-fold, respectively, after the high dose. In nasal secretions the specific IgA and IgG increased 2- and 6-fold after the medium dose and 2- and 20-fold after the high dose, respectively. In vaginal secretions the specific IgA and IgG increased 3- and 5-fold after the medium dose and 56- and 74-fold after the high dose, respectively. The lowest dose did not induce any significant antibody titer increases in serum or in secretions. The specific IgA and IgG levels in secretions were still elevated after 6 months but were decreasing 1 year after the vaccination. These results show that intranasal vaccination of humans with CTB induces strong systemic and mucosal antibody responses and suggest that CTB may be used as a carrier for antigens that induce protective immunity against systemic as well as respiratory and genital infections.

Mucosal vaccination leads to local production of antibodies and immunologic memory, which are important in the defense against many pathogens that colonize the surfaces in the gastrointestinal, respiratory, and urogenital tracts (18). Local production of immunoglobulin A (IgA) antibodies in the intestine after administration of microbial antigens perorally has been studied extensively, and this has resulted in the development of several oral human vaccines (19). According to the concept of the common mucosal immune system, immunocytes induced in the gut-associated lymphoid tissues migrate not only to the entire intestine but also to mucosal tissues of other organs (29). However, a compartmentalization of the mucosal immune system has been suggested, since oral, nasal, and rectal routes of vaccination induced greater immune responses at sites proximal to the site of induction than at distal sites (20, 35).

There is currently no doubt about the function of the respiratory tract as a good inductive site for mucosal as well as systemic antibody responses in experimental animals, but this knowledge has been poorly exploited for humans. Although a few studies have shown positive results with influenza virus, no human vaccines are routinely given intranasally or by inhalation (6, 13). Attempts have also been made to induce local antibody production by intravaginal immunization in animals, but both mucosal and systemic responses have been rather modest and dependent on the stage of the reproductive cycle (10, 30, 33). Intravaginal immunization of human volunteers resulted in a local antibody response which was greater than that after peroral immunization (43). However, animal experiments have shown that intranasal immunization induces a specific antibody response in the genital tract, although the mechanisms behind the immunologic connection between such anatomically and functionally remote organs are unknown (10, 12, 20, 38).

Most protein and polysaccharide antigens are poor mucosal immunogens, partly owing to their lack of receptor-binding properties (1). Nontoxic exceptions are antigens such as cholera toxin B subunit (CTB), *Escherichia coli* heat-labile enterotoxin (LT) B subunit, and genetically detoxified pertussis toxin (10, 17, 36). CTB, which binds to the GM1 ganglioside, has been used as a mucosal antigen in several animal models and is also a constituent of an oral cholera vaccine for human use (17, 18). CTB has also been employed as a mucosal carrier for nonbinding protein or polysaccharide antigens. Such constructs have been given orally, intranasally, or intravaginally to different species of animals but not to humans (2, 3, 8, 30, 38).

The objective of the present study was to assess the safety and immunogenicity of CTB given intranasally to humans and to evaluate whether such local immunization may elicit IgA and IgG antibody production not only on the nasal mucosa but also in serum and in the female genital tract.

MATERIALS AND METHODS

Vaccine. The vaccine consisted of purified CTB provided by SBL Vaccine, Stockholm, Sweden, and is a constituent of a licensed oral vaccine against cholera. The CTB was produced and purified from a recombinant strain of *Vibrio cholerae* lacking the cholera toxin A subunit gene (39). The vaccine was diluted in phosphate-buffered saline (PBS) to the different concentrations used. The content of endotoxin was 10 ng/mg of CTB protein.

Subjects and vaccination. Forty-five healthy Swedish volunteers of both sexes (24 female and 21 male), aged 19 to 35 years, gave informed consent to participate in the study, which was approved by the Swedish Medical Products Agency and the local Human Research Ethical Committee of the Medical Faculty, Göteborg University, Göteborg, Sweden. None of the volunteers had been vac-

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cinated against cholera or had traveled during the last 3 years to a country where enterotoxigenic *E. coli* or cholera is prevalent.

The intranasal CTB vaccine was given to three groups of volunteers in three different doses, i.e., 10, 100 or 1,000 μg/dose. Nineteen subjects received the 10-μg dose and 20 received the 100-μg dose, but because of adverse reactions, only six received the 1,000-μg dose. Two doses were given with a 2-week interval, and the vaccine was administered as a 100-μl spray given twice in both nostrils, i.e., a total volume of 400 μl, with an atomizer (Apoteksbolaget AB, Göteborg, Sweden; a kind gift from Astra-Draco, Lund, Sweden). The CTB concentration did not decrease as a result of the passage through the atomizer as measured by single radial immunodiffusion (27).

Follow-up for adverse reactions. All subjects underwent a physical examination of the nasal and oral mucosa before and 1 week after vaccination and were questioned about ongoing allergic reactions, frequent nasal bleedings, and previous tonsillectomy ($n = 4$) or adenoidectomy ($n = 2$). Surveillance for possible side effects was performed during five consecutive days after each vaccination. A questionnaire inquiring about the occurrence of symptoms that might be related to vaccination (nasal pain or itching, sneezings, nasal congestion, increased nasal secretions, nasal bleeding, dizziness, temperature, rash, and other reactions) was completed each day by each of the volunteers.

Sampling of sera and secretions. Blood and nasal and vaginal secretion samples were taken immediately before the first vaccination and 1 week after the second vaccination. Samples were taken after 6 months ($n = 16$) and 1 year ($n = 14$) from volunteers in the medium- and high-dose groups. The nasal secretions were sampled by using a tampon method. A dry double cotton gauze (1.5 by 20 cm) (Scholl, Solna, Sweden) was inserted into one of the nostrils with a pair of tweezers and a nasal speculum and left in place for approximately 2 h. After removal, the tampon was placed in 1.0 ml of PBS and stored at −70°C. The tampons were thawed, and the fluid was squeezed out by centrifugation (2,200 × *g*) in a pierced Eppendorff tube placed on top of another tube and was immediately analyzed. The vaginal secretions were sampled by using a Polywick tampon (10 by 30 mm) (Polyfiltronics Group Inc., Rockland, Mass.) (14, 21), which was inserted deeply into the vagina by the female volunteer herself. After 2 h, the tampon was taken out by the volunteer with the aid of a thread attached to the Polywick, placed in 1.0 ml of PBS, and stored at −70°C. The Polywicks were thawed and centrifuged for 10 min at 4,500 × *g* in pierced Eppendorff tubes as described above, and the samples were treated with bromelain (Sigma Chemical Company, St. Louis, Mo.) to solubilize the mucus (43). Twenty-five micrograms of bromelain was added per ml of sample and incubated for 60 min at 37°C, followed by centrifugation for 10 min at 9,500 × *g*.

Determination of total Ig and specific antibodies. The total IgA, total secretory IgA (SIgA), and total IgG antibody contents in sera and secretions were determined by enzyme-linked immunosorbent assay (ELISA) in polystyrene microtiter plates (Nunc, Roskilde, Denmark). Goat anti-human IgA (α-chain specific) (catalog no. 109-005-011; Jackson ImmunoResearch Laboratories, West Grove, Pa.) or goat anti-human IgG $(Fab)_2$ (catalog no. 109-005-097; Jackson) was used for coating the microtiter wells (1 μg/ml) at room temperature overnight. After blocking of the plates with 0.1% bovine serum albumin–PBS, the samples and references were added in threefold serial dilutions. As references we used polyclonal human plasma IgA (catalog no. 401098; Calbiochem Corp., La Jolla, Calif.), SIgA purified from colostrum (catalog no. P020; Nordic Immunological Laboratories, Tilburg, The Netherlands) (used in SIgA ELISA only), and polyclonal human plasma IgG (catalog no. 40114; Calbiochem), respectively, added at 400 ng/ml to the first well. After incubation at room temperature for 2 h, bound total IgA, total SIgA, and total IgG antibody were demonstrated by incubating the plates for 2 h with horseradish peroxidase (HRP)-conjugated goat anti-human serum IgA (α-chain specific) (catalog no. 109-035-011; Jackson), HRP-conjugated goat anti-human secretory component (SC) (catalog no. 67124; Nordic), and HRP-conjugated goat anti-human IgG, (Fcγ specific) (catalog no. 109-035-008; Jackson), respectively. The plates were developed by using *o*-phenylenediamine and H_2O_2, and the endpoint titers were determined as the reciprocal dilution giving an absorbance at 450 nm of 0.4 U above the background level (Labsystems Multiscan PLUS) after 20 min of enzyme reaction. All titrations were performed in duplicate.

The CTB-specific antibodies were determined by a modified GM1 ELISA (41). Plates were coated with 0.3 μM GM1 in PBS at room temperature overnight. After blocking of the plates with 0.1% bovine serum albumin–PBS, they were incubated with 0.5 μg of CTB per ml. The samples, a positive serum reference, and a positive nasal secretion reference were added in threefold dilutions and incubated for 2 h at room temperature. Bound CTB-specific IgA, SIgA, and IgG antibodies were demonstrated as described above. The titers obtained from different plates were adjusted according to the positive serum reference or the positive nasal secretion reference, as appropriate. Samples with titers below the detection limit were assigned a titer of one-half of the lowest dilution. The specific antibody content in secretions was expressed as arbitrary units per milliliter. The CTB-specific IgA, SIgA, and IgG antibody contents were divided by the total IgA, SIgA, and IgG concentrations (micrograms per milliliter) in the nasal and vaginal secretion samples to adjust for variations in the Ig content in secretion sample eluates collected from different volunteers and on different days. The fold increases were calculated by dividing the adjusted postvaccination value by the adjusted prevaccination value from each individual. On the basis of calculations of the methodological errors for the different ELISAs used, a greater than twofold increase was chosen to define the volunteers who responded to the vaccine (22).

The total IgE content in serum was determined by a paper radioimmunosorbent test with a commercially available kit (Pharmacia, Uppsala, Sweden). The presence of specific IgE was tested by a GM1 ELISA, as described above, with HRP-conjugated goat anti-human IgE (ε-chain specific) (catalog no. 67112; Nordic) as the secondary antibody.

Statistical methods. Before calculations, all secretion titers were adjusted for total Ig content, and all values were $\log_{10}$ transformed. The variation of the specific titers was shown as the range (geometric mean [GM] ± standard error of the mean [SEM]). Confidence intervals (95%) were given for the total IgA and IgG levels in secretion sample eluates. Analysis of variance was used as appropriate for analysis of the significances of differences in titers, and post-hoc comparisons of the individual groups were performed with Scheffé's test. The software Statistica 4.0 for Windows (Softstat, Tulsa, Okla.) was used for calculations. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Adverse reactions. To evaluate the feasibility of vaccinating humans intranasally with future CTB conjugates, we carefully recorded all signs and symptoms of adverse reactions. All volunteers in the 1,000-μg dose group experienced profuse nasal secretion, itching, and violent sneezings after the second vaccination. Four of them had similar reactions also after the first dose, and one of these volunteers withdrew. None of these reactions lasted for more than 1 day, and there were no systemic reactions, but the high frequency of local side effects prompted us to discontinue recruitment of additional volunteers to this dose group. In the 100-μg dose group, 7 of 20 volunteers reported adverse reactions that were of the same type as described above but very mild and of much shorter duration. None of the volunteers in the 10-μg dose group experienced any adverse reactions. The physical examination of the nasal mucosa before and after vaccination showed that there were no visible effects of CTB irrespective of the dose given.

Serum antibody responses. CTB-specific IgA and IgG antibody titers in sera of all volunteers were determined. The GMs of the titers in the different groups before the first vaccination and 1 week after the second vaccination are shown in Fig. 1. The 100- and 1,000-μg doses induced impressive increases in the titers of anti-CTB IgA ($P < 0.001$ in both cases) and IgG ($P < 0.001$ in both cases), but no significant titer increases were seen in the 10-μg dose group. The fold increases of the CTB-specific IgA in the 100- and 1,000-μg dose groups were 21.2 and 61.4, respectively, and those for the IgG were 7.4 and 37, respectively. In the high-dose group all volunteers responded (>2-fold) with both specific IgA and IgG, and in the medium-dose group 18 of 20 and 17 of 20, respectively, responded. In the low-dose group only 8 of 18 volunteers responded with IgA, and 6 of 18 responded with IgG. The level of the preimmune CTB titers did not seem to influence the response to CTB (data not shown). We found no specific IgM titer increases irrespective of dose (data not shown).

To test whether intranasal vaccination with CTB induces allergic reactions associated with IgE production, we measured total IgE and specific IgE anti-CTB antibodies in serum before vaccination and 1 week after vaccination in all individuals in the high-dose group and in five of the volunteers in the medium-dose group. However, there was no increase in total IgE in any of these individuals with or without adverse reactions, nor could we detect any CTB-specific IgE in any of these 10 volunteers (data not shown).

Nasal antibody responses. Nasal secretions were collected by using a tampon method, which was shown to give a sufficiently high yield of antibodies (GM [95% confidence interval] 173 [149 to 203] μg/ml for total IgA and 199 [167 to 236] μg/ml for total IgG) to also allow detection of low levels of specific

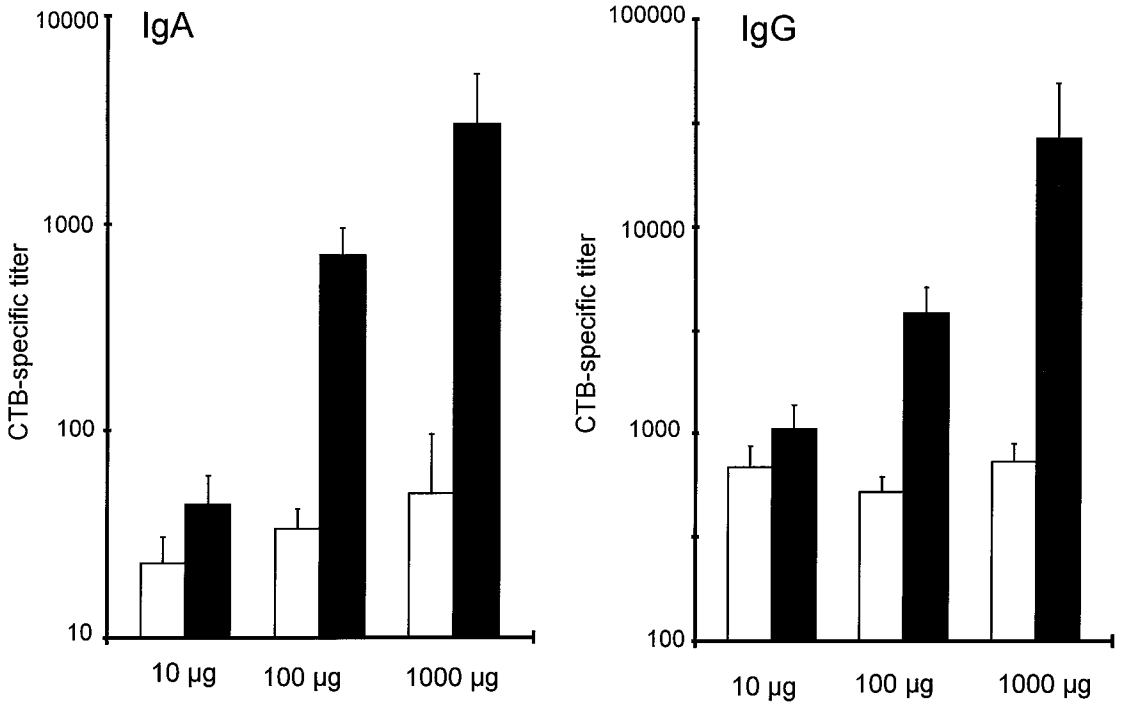

FIG. 1. CTB-specific IgA and IgG titers in serum before (empty bars) and 1 week after (filled bars) the second vaccination with either, 10, 100 or 1,000 μg per dose. Error bars show SEMs.

antibodies in sample eluates. The levels of total IgA and IgG did not differ before and after the vaccination with CTB (data not shown). The pre- and postvaccination titers, adjusted to the total IgA or IgG content, are shown in Fig. 2, and the fold increases and frequency of responders are shown in Table 1.

To emphasize that the IgA and IgG titer increases in nasal secretions were not due to variations in total Ig, we show both the adjusted titers (Fig. 2) and the unadjusted titers (Table 1). The anti-CTB IgA titers in the nasal secretions were augmented moderately by vaccination with the 100- and 1,000-μg

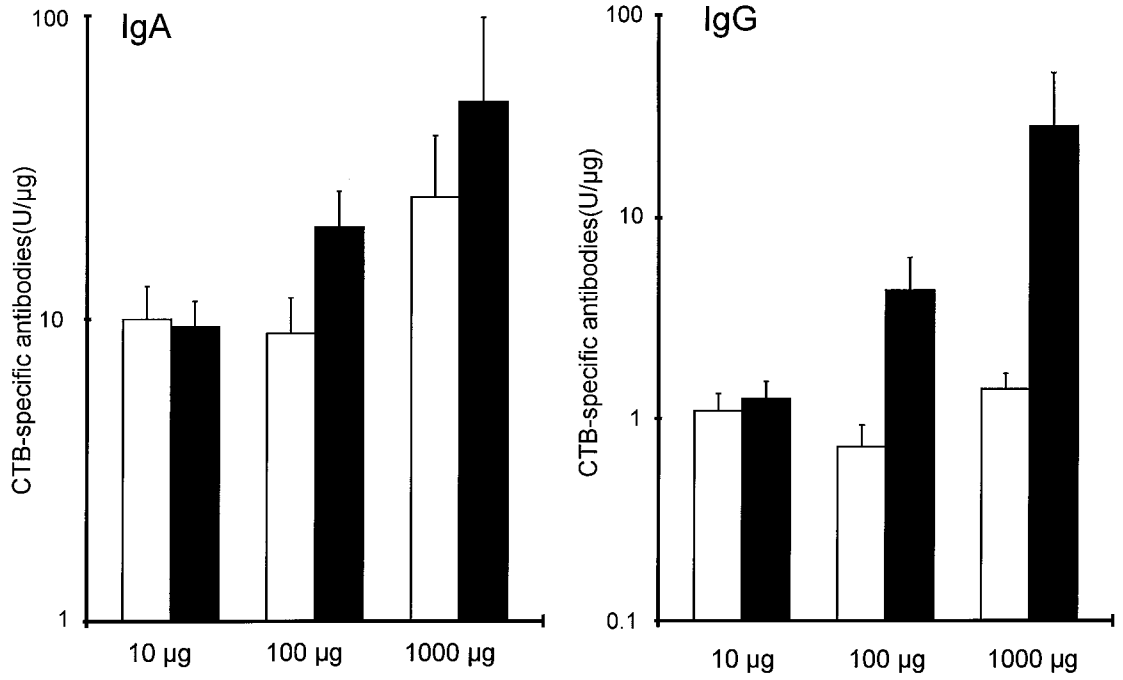

FIG. 2. CTB-specific IgA and IgG (units per microgram of total Ig) in nasal secretions before (empty bars) and 1 week after (filled bars) the second vaccination with either, 10, 100, or 1,000 μg per dose. Error bars show SEMs.

TABLE 1. Antibody responses against CTB in nasal secretions after intranasal vaccination

Ig and dose (μg)	GM of CTB-specific titer[a] on day:		Frequency of responders[b]	GM of fold increase[c] for:	
	0	21		All subjects	Responders
IgA					
10	198 (140–280)	155 (117–206)	3/18	1.0	2.6
100	156 (110–221)	261 (190–359)	8/20	2.3	9.7
1,000	746 (457–1,214)	1,263 (706–2,259)	3/5	2.0	5.2
IgG					
10	23 (18–30)	28 (22–35)	6/18	1.1	3.1
100	14 (11–17)	74 (50–110)	14/20	6.0	12.7
1,000	29 (21–39)	525 (234–1,173)	5/5	20.2	20.2

[a] The range (±1 SEM) is given in parentheses.
[b] Responders were defined as having a >2-fold titer increase.
[c] The fold increases are calculated from the titers adjusted for total Ig content in the sample.

doses, but the increases were not statistically significant (Fig. 2). At least part of the specific IgA found in nasal secretions was SIgA, since SC was detected in all but one postvaccination sample in the GM1 ELISA (data not shown). In contrast to the IgA titers, the IgG titers increased significantly in both the 100- and 1,000-μg dose groups ($P < 0.001$ and $P = 0.002$, respectively). The frequency of IgG responders was also higher than the frequency of IgA responders (Table 1). No specific IgM titer increases were found irrespective of dose (data not shown).

Vaginal antibody responses. Vaginal secretions were collected by using a Polywick tampon. As the total levels of IgA and IgG in pre- and postvaccination samples varied in some of the individuals, the specific antibody titers were adjusted to the total amounts of IgA and IgG, respectively. The GM (95% confidence interval) of the total IgA from all diluted (approximately 1/6) vaginal sample eluates was 16 (10 to 25) μg per ml, and that of the total IgG was 149 (100 to 217) μg per ml. The pre- and postvaccination titers are shown in Fig. 3, and the fold increases and frequency of responders are shown in Table 2. Similarly to the case for the nasal samples, the IgA and IgG titer increases in vaginal secretions were not due to variations in total Ig. To stress this, we show both the adjusted titers (Fig. 3) and the unadjusted titers (Table 2). The 10-μg dose did not induce any IgA or IgG increases, but the 100- and 1,000-μg doses induced considerable IgA and IgG increases in most individuals. Although both the IgA and IgG titers were augmented in the high-dose group, only the IgA increase was statistically significant ($P = 0.013$ and 0.06, respectively), because of the low numbers of individuals in the groups and the large difference in the magnitude of the adjusted titers between different individuals. All individuals in the 1,000-μg dose group responded with both IgA and IgG. Seven of nine individuals in the 100-μg dose group responded with either IgA or IgG. No significant IgM increases were found in any of the groups (data not shown). The presence of SC in the vaginal secretions thus indicated that at least part of the CTB-specific IgA is SIgA (data not shown).

Antibody responses 6 months and 1 year after vaccination. To determine the persistence of the antibody responses in serum and secretions, samples were taken from 16 volunteers 6 months after vaccination and from 14 of these volunteers after 1 year. The specific IgA titer in serum had declined significantly after 6 months ($P < 0.001$), but the specific IgG titer did not change significantly (Fig. 4). Both IgA and IgG levels were still elevated after 1 year compared with the prevaccination levels.

We found that both IgA and IgG CTB-specific titers (adjusted for the total content of IgA and IgG) in nasal secretions

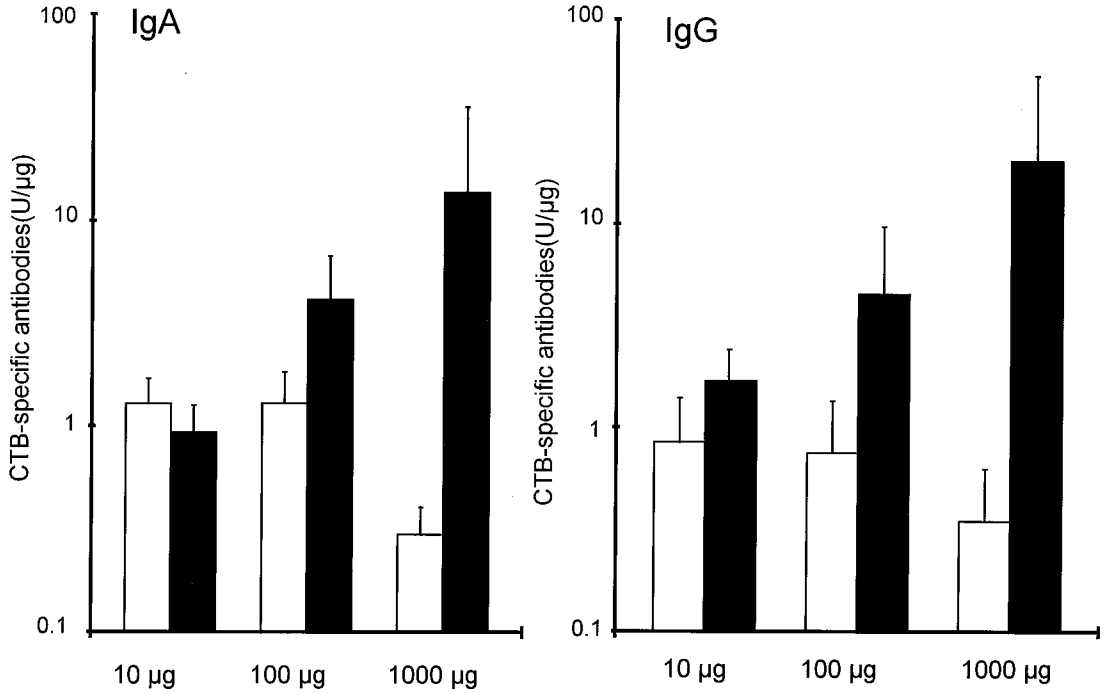

FIG. 3. CTB-specific IgA and IgG (units per microgram of total Ig) in vaginal secretions before (empty bars) and 1 week after (filled bars) the second vaccination with either, 10, 100, or 1,000 μg per dose. Error bars show SEMs.

TABLE 2. Antibody responses against CTB in vaginal secretions after intranasal vaccination

Ig and dose (μg)	GM of CTB specific titer[a] on day:		Frequency of responders[b]	GM of fold increase[c] for:	
	0	21		All subjects	Responders
IgA					
10	<2	<2	0/7		
100	2.4 (1.5–3.8)	15 (7.8–29)	6/9	3.2	21.1
1,000	1.2 (1.0–1.41)	40 (17–98)	4/4	55.5	55.5
IgG					
10	12 (7.4–20)	24 (17–35)	3/7	1.9	3.6
100	11 (6.0–19)	65 (30–140)	6/9	5.5	13.9
1,000	5.0 (2.8–9.0)	295 (113–768)	4/4	74	74

[a] The range (±1 SEM) is given in parentheses.
[b] Responders were defined as having a >2-fold titer increase.
[c] The fold increases are calculated from the titers adjusted for total Ig content in the sample.

had increased significantly 6 months after vaccination compared to titers obtained after 1 week ($P = 0.005$ and 0.01, respectively) (Fig. 5). The long-term persistence of the nasal antibody responses was unexpected, particularly as the serum IgA had decreased substantially. However, 1 year after vaccination, the CTB-specific IgA and IgG titers had declined compared with the titers obtained 6 months after vaccination. However, the IgG titers were still elevated compared with the prevaccination levels.

In contrast to the CTB-specific IgA and IgG titers in the nasal secretions, which increased, and to the IgA titers in serum, which decreased, the IgA and IgG titers (adjusted for the total content of IgA and IgG) in vaginal secretions were maintained at the same level after 6 months as after 1 week (Fig. 6). After 1 year, the CTB-specific IgA and IgG titers had declined significantly compared with the values 1 week after the vaccination ($P = 0.0011$ and 0.03, respectively).

DISCUSSION

Recent animal experiments have indicated the possibility of using protein antigens as vaccines or vaccine carriers administered locally on the respiratory mucosa to induce immune responses at local and distant mucosae as well as systemically (10, 20, 38). This is the first study to show that the potential mucosal carrier CTB given intranasally may elicit IgA and IgG antibody production not only on the nasal mucosa and in serum but also in the human female genital tract. Moreover, the dose of CTB that can safely be given intranasally to humans with no or tolerable side effects was determined.

Our results show that intranasal vaccination of humans gives rise to impressive serum antibody responses of both specific IgA and IgG. As recombinant CTB (rCTB) is a component of the oral cholera vaccine, it is possible to compare the magnitudes of the fold increases in serum after intranasal vaccination

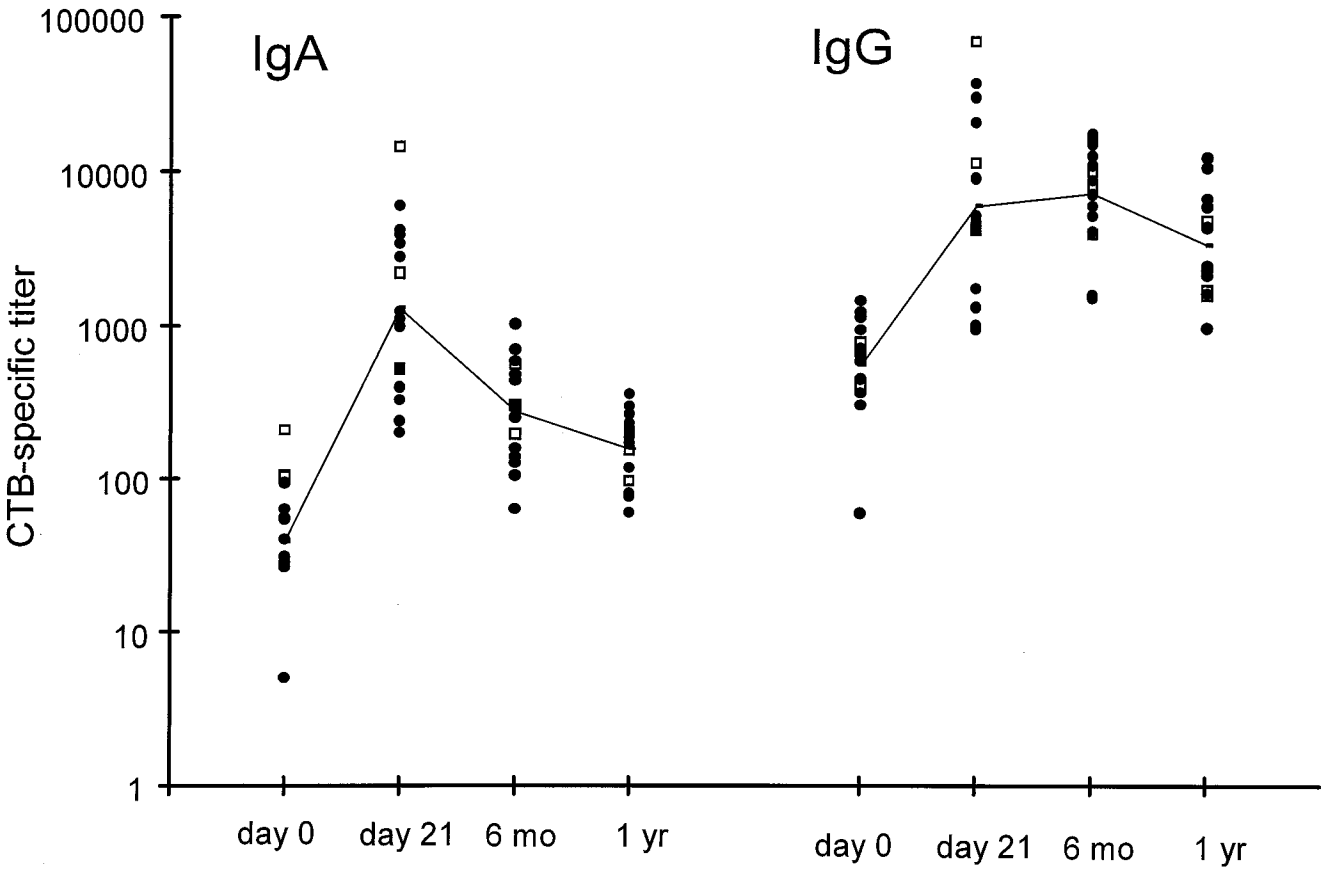

FIG. 4. Persistence of the CTB-specific IgA and IgG titers in serum. Filled symbols, individuals given 100 μg per dose; open symbols, individuals given 1,000 μg per dose; connected points, GMs. mo, months; yr, year.

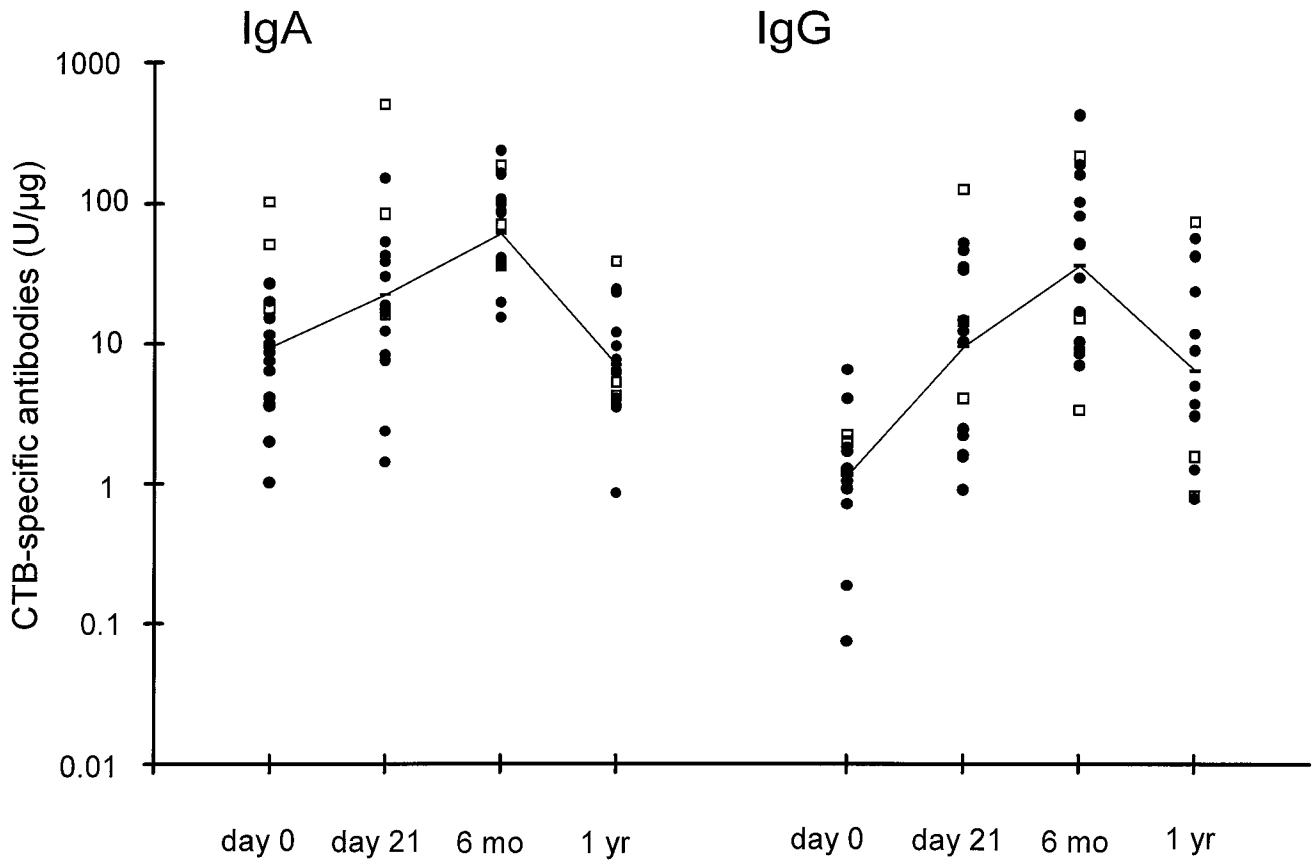

FIG. 5. Persistence of the CTB-specific IgA and IgG (units per microgram of total Ig) in nasal secretions. Filled symbols, individuals given 100 μg per dose; open symbols, individuals given 1,000 μg per dose; connected points, GMs. mo, months; yr, year.

with those after peroral vaccination (23). Giving the same dose by the nasal or oral route resulted in about three-times-higher fold increases of specific serum IgA or IgG after intranasal vaccination than after oral vaccination. Administration of a 10-times-lower dose intranasally resulted in magnitudes of serum IgA and IgG increases similar to those with the peroral vaccine. This indicates that the intranasal route may be more effective in inducing systemic responses than the oral route, which is supported by animal studies using other antigens coupled to CTB given by various routes (3, 16, 44).

We found that intranasal vaccination with both the medium and high doses of CTB induced substantial IgA and IgG titer increases in vaginal secretions already 1 week after vaccination. The persistence of the antibody response in vaginal secretions 6 months after vaccination is noteworthy, since mucosal responses are generally regarded as short-lived. Although local application of various antigens in the vagina has been shown to elicit a specific IgA antibody response in the vaginal secretions in both humans and animals (26, 43), intranasal immunization generally induced a higher vaginal response than intravaginal administration in animals (10, 12, 20). The advantages of immunizing intranasally may be not only a higher efficacy in eliciting a vaginal immune response but also a more effective induction of a systemic immune response and a more convenient route of administration. Moreover, it might be difficult to use intravaginal immunization in humans if the time point for vaccination has to be synchronized to the menstrual phase, as suggested by results showing that induction of immune responses in the mouse vagina correlates with the stage in the estrous cycle (20, 33). Intranasal vaccination is also applicable to both sexes and might induce an immune response in the male urogenital tract, which has not yet been evaluated.

A few studies with animals suggest that oral vaccination may also induce local immunity in the genital tract (7, 14, 20, 30). However, several of these studies indicate that peroral vaccination is not as efficient as intranasal vaccination in inducing a specific response in the vaginal secretions (20, 30). Notably, a study comparing oral and intravaginal vaccination with a whole-cell cholera vaccine containing rCTB in humans showed that oral vaccination does not induce significant responses against CTB in the vaginal secretions, despite the appearance of a strong serum response in some of the volunteers (43). Moreover, since experiments with animals have indicated that specific IgA and IgG antibody-producing cells appear in the vaginal tissues after intranasal immunization (25, 37), it is very likely that the majority of the IgA antibodies are locally produced and not transudated from serum. The origin of the specific IgG in vaginal secretions is probably both cells in the vaginal tissue and serum, since specific IgG antibodies against tetanus toxoid have been found in human vaginal secretions after parenteral vaccination (4).

In the common mucosal immune system, lymphocyte precursors are induced in inductive mucosal lymphoid tissues, enter the circulation, and end up in various mucosal and systemic effector sites (29, 31). The mechanism for the appearance of lymphocytes in the genital tract after immunization in the airways is not clear, but it may be a result of the existence of common adressins for lymphocyte homing receptors in the

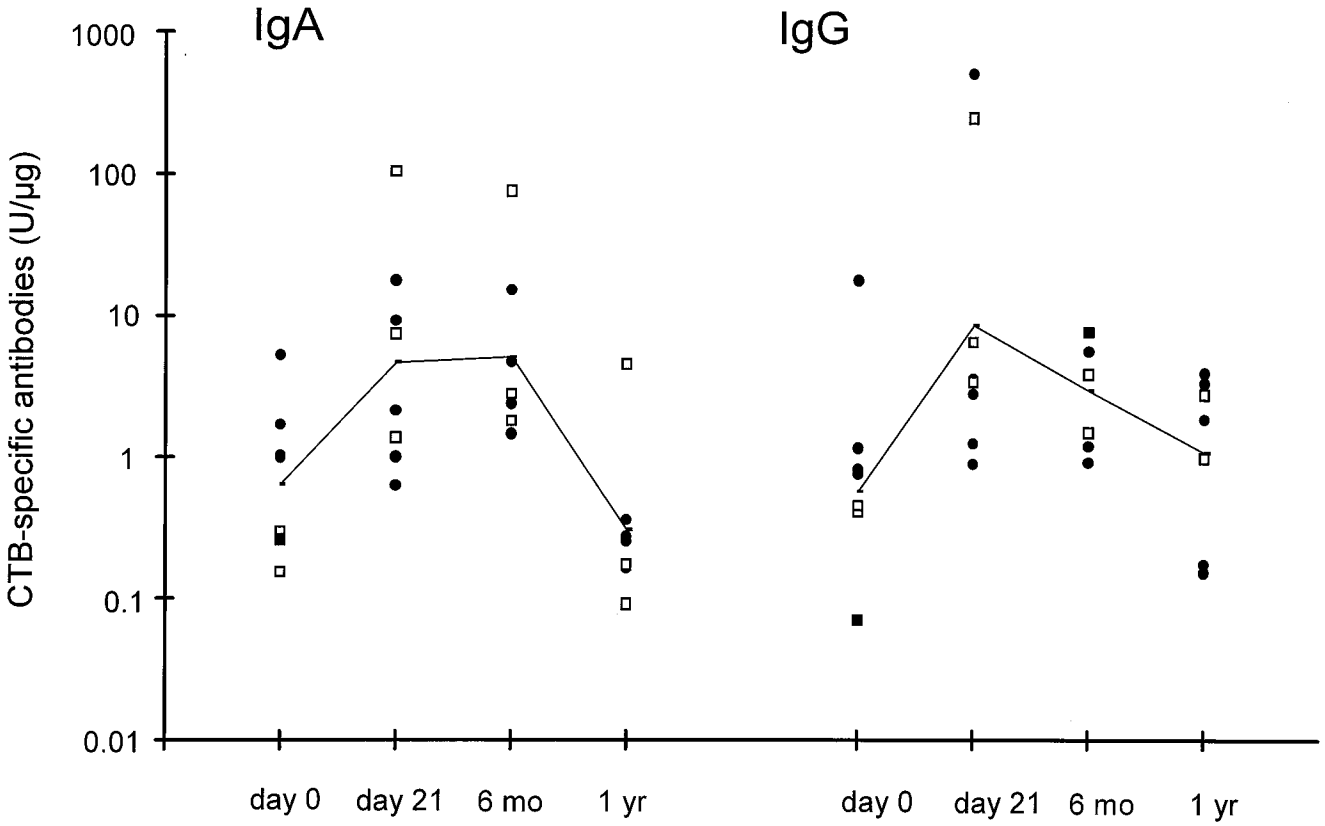

FIG. 6. Persistence of the CTB-specific IgA and IgG (units per microgram of total Ig) in vaginal secretions. Filled symbols, individuals given 100 μg per dose; open symbols, individuals given 1,000 μg per dose; connected points, GMs. mo, months; yr, year.

two disparate tissues. However, the lymphocyte receptors or endothelial adressins responsible for lymphocyte homing to the respiratory and the genital tracts have not yet been characterized.

It has been established in studies with both animals and humans that intranasal immunization elicits an antibody response in the upper respiratory tract (3, 11, 13, 15, 35). Our results showing that the CTB-specific IgG response dominated over the IgA response in the nose indicate that the induction of antibody isotypes to CTB in the upper respiratory tract is different from the response in the intestinal tract (34). In keeping with our results, a previous study showed that the predominant total Ig-secreting cell population in the tonsils and adenoids secreted IgG and not IgA (35), and intranasal vaccination of humans with influenza virus induces a higher proportion of IgG-secreting cells than IgA-secreting cells in blood (32). Moreover, immunohistochemical examinations of the nasal mucosa have shown that substantial numbers of IgG-producing cells are present in the stroma beneath the surface epithelium (5). Since enzymatic degradation of IgG in the respiratory tract is probably a minor problem compared with that in the gastrointestinal tract, the produced IgG may be an important contributor to protection (28). We were surprised by the relatively modest increases of specific IgA titers in the nasal secretions 1 week after the vaccination. However, the maximal response might occur later than 1 week after the vaccination, since the specific IgA titers had increased further after 6 months. It has been reported that intranasal vaccination of humans with live influenza virus induced a specific IgA response that was delayed compared with that after oral vaccination (32). We are currently investigating the kinetics of the anti-CTB antibody responses after intranasal and peroral vaccination.

Although the rCTB preparation that we have used is completely free of CT and contains low levels of endotoxin, the administration of 1,000 μg per dose induced irritating local adverse reactions in all of the volunteers. In spite of the relatively short duration of the local reactions and the absence of systemic reactions, their existence would appear to preclude the practical use of this dose intranasally in a vaccine formulation. About one-third of the volunteers also experienced local adverse reactions with 100 μg of rCTB per dose, but these reactions were very mild and were considered tolerable by the affected individuals. In a recent study, 100 μg of the B subunit of LT of enterotoxigenic *E. coli* with trace amounts of LT was given intranasally to humans and resulted in a high frequency of both local and general reactions, which indicates that the LT component is very reactogenic (15). Thus, the necessity of using completely detoxified proteins in humans is obvious. As the adverse effects were very mild after the 100-μg dose of rCTB, a higher dose would probably be tolerated and would perhaps increase the immune response even further. A CTB dose of 330 μg has in fact been given to humans in a recent study, without any reported adverse effects (9). Because of the reported effect of CT or CTB with trace amounts of CT to induce IgE antibodies against some protein antigens when administered perorally or intranasally to mice (40, 42), we were interested in whether rCTB would induce an increase in total or CTB-specific IgE after intranasal administration to humans. However, this was not the case, since we found no elevations in either the total or CTB-specific IgE antibody content in serum. Similar results were also found after peroral vaccination with

the whole-cell rCTB cholera vaccine (24). Thus, the systemic and severe local side effects as well as stimulation of IgE production in the studies mentioned above seem to be caused by the holotoxins.

The present study is the first to show that intranasal vaccination elicits specific IgA and IgG antibodies in the vaginal secretions of humans. The effective induction and long-term persistence of systemic antibodies as well as local antibodies in the upper respiratory tract and vagina suggest that rCTB may be used as a vaccine carrier for antigens from pathogens with either the respiratory, genital, or systemic site of entry. In future studies we will evaluate whether intranasal vaccination may elicit a specific antibody response also in bronchoalveolar lavage fluids and in the male urogenital tract.

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